DECLARATION

I, Masanori Hirota of HIROTA AND ASSOCIATES, residing at Wakabayashi Bldg. 3F, 8-5, Akasaka 2-chome, Minato-ku, Tokyo 107-0052, Japan, do hereby certify that I am conversant with the English and Japanese languages and am a competent translator thereof, and I further certify that to the best of my knowledge and belief the following is a true and correct translation made by me of the document in the Japanese language filed for a patent application in Japan under No. 11/228,282 on August 12, 1999 in the name of JAPAN SCIENCE AND TECHNOLOGY CORPORATION at Tokyo, Japan, entitled: "BACTERIAL CELL WALL COMPONENT-UNRESPONSIVE MODEL MOUSE".

Signed this 12 day of May, 2003

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[Title of the Invention] BACTERIAL CELL WALL COMPONENT-UNRESPONSIVE MODEL MOUSE

[Scope of Claims]

[Claim 1] A bacterial cell wall component-unresponsive non-human animal characterized by that its function of myeloid differentiation primary response gene is deficient on its chromosome.

[Claim 2] The non-human animal according to claim 1, wherein the bacterial cell wall component is endotoxin derived from Gram-negative bacteria.

[Claim 3] The non-human animal according to claim 1, wherein the bacterial cell wall component is a cell wall component of Gram-positive bacteria.

[Claim 4] The non-human animal according to claim 3, wherein the cell wall component of Gram-positive bacteria is peptidoglycan deived from Gram-positive bacteria, lipoteichoic acid or Mycobacterium tuberculosis lysate.

[Claim 5] The non-human animal according to claim 4, wherein the non-human animal is a rodent.

[Claim 6] The non-human animal according to claim 5, wherein the rodent is a mouse.

[Claim 7] An assessing method of a subject material characterized in comprising the steps of: the subject material is administered to the non-human animal being deficient in function of myeloid differentiation primary response gene; an endotoxin activity of the subj ct material is assessed.

[Claim 8] An assessing method of a subject mat rial characterized in comprising the steps of: the subject material is administered to the non-human animal being deficient in function of myeloid differentiation primary response gene; an interleukin-1 activity of the subject material is assessed.

[Claim 9] An assessing method of a subject material characterized in comprising the steps of: the subject material is administered to the non-human animal being deficient in function of myeloid differentiation primary response gene; an interleukin-18 activity of the subject material is assessed.

[Claim 10] An assessing method of a subject material characterized in comprising the steps of: the subject material is administered to the non-human animal being deficient in function of myeloid differentiation primary response gene, and a wild-type non-human animal of the non-human animal; an endotoxin activity of the subject material is assessed.

[Claim 11] An assessing method of a subject material characterized in comprising the steps of: the subject material is administered to the non-human animal being deficient in function of myeloid differentiation primary response gene, and a wild-type non-human animal of the non-human animal; an interleukin-1 activity of the subject material is assessed.

[Claim 12] An assessing method of a subject material characterized in comprising the steps of: the subject material is administered to the non-human animal being deficient in function of myeloid differentiation primary r sponse gene, and a wild-type non-human animal of th non-human animal; an

interleukin-18 activity of the subject material is assessed.

[Claim 13] The assessing method of a subject material according to any one of claims 7 to 12, wherein the non-human animal is a mouse.

[Claim 14] A knockout mouse characterized in being unresponsive to a bacterial cell wall component.

[Claim 15] The knockout mouse according to claim 14, wherein the bacterial cell wall component is a cell wall component of Gram-positive bacteria.

[Claim 16] A method of detecting bacterial cell wall components characterized in comprising the steps of: a subject material is administered to a knockout mouse being unresponsive to bacterial cell wall components; bacterial cell wall components in the subject material are detected.

[Claim 17] A method of detecting bacterial cell wall components characterized in comprising the steps of: a subject material is administered to a knockout mouse being unresponsive to bacterial cell wall components and a wild-type mouse; bacterial cell wall components in the subject material are detected.

[Detailed Description of the Invention]

[0001]

[Technical Field to Which the Invention Pertains]

The present invention relates to bacterial cell wall component-unresponsive non-human animals being deficient in function of myeloid differentiation primary response (hereinafter "MyD88") g ne, and particularly relates to MyD88

knockout mice and assessing methods of an ndotoxin activity, an interleukin-1 activity, an interleukin-18 activity and the like in subject materials with the MyD88 knockout mice.

[0002]

[Prior Art]

Cytokines are intracellular signal transmitters which play an important role in an immune response, a response upon infection, hematopoiesis, inhibition of virus infection and tumor cells. Among them, a cytokine which transmits signals between lymphocytes is called interleikin (hereinafter, "IL"). Among ILs, IL-1 is a cytokine which mediates various immune responses and inflammatory responses, and is involved in maintenance of homeostasis of living organisms and produced from various cells such as monocytes, macrophages, keratinocytes, vascular endothelial cells and the like when the living organisms get infected or hurt. It has been known that there are two kinds of IL-1, IL-1α and IL-1β, both of which combine to the same receptor. It has been also known that IL-1 exerts its function simultaneously with the activation by an antigen to T cell and by mitogens, makes T cells release IL-2, and enhances the expression of IL-2 receptors to induce T cell proliferation, and that it acts on monocytes and macrophages in order to induce the production of TNF, IL-1, IL-6.

[0003]

IL-1 has two kinds of IL-1 receptors (hereinafter "IL-1R"), and both of the IL-1Rs, typ I and type II, have three immunogloblin-like domains in their extracellular domains.

Type I receptors express in T cells and connective tissue, and type II receptors express in splenic B cells, myeloids and the like, and it has been known that type I receptors induce NF-$_{\kappa}$B in nuclei. It has been also known that there is an IL-1 receptor antagonist (hereinafter "IL-1ra") which shows no bioactivity in spite that it binds to IL-1R with the affinity similar to that of IL-1α and IL-1β, and that it prevents IL-1 from binding to IL-1R competitively.

[0004]

IL-18 is known to promote the production of interferon-γ (hereinafter "IFN-γ"), to enhance the activation of natural killer cells, to induce the production of IFN-γ from T cells in cooperation of IL-12, and to act an important role in a Th1 (IL-2 producting helper T cells) response. Further, it is known that IL-18 has no structural similarity to IL-12 in spite that it has a functional similarity, and has a structural similarity to IL-1. Moreover, it has been also known that IL-18 is produced as an inactive precursor that requires cleavage by IL-1β-converting enzyme (ICE)/caspase1 for its maturation, as in the case of IL-1β, and that IL-18 activates IL-1R-associated kinase (IRAK) and NF-$_{\kappa}$B.

[0005]

A plurality of molecules showing homology to IL-1R have been identified so far, and signal pathways mediated by IL-1R family is being studied intensively now. It has been known that MyD88 is a cytoplasmic protein comprised of an IL-1R homologous domain and a Death domain, and functions as an

adaptor molecule which activates NF-$_{\kappa}$B by recruiting IRAK to IL-1R complex after IL-1 stimulation, and that MyD88 gene was originally separated as a myeloid differentiation primary response gene, which rapidly induces M1 myeloleukemic cells to macrophages by IL-6-stimulated differentiation.

[0006]

Toxins in bacterial cells being comprised of lipopolysaccharide, which is a major structural component of the outer membrane encompassing peptidoglycan on the surface of Gram-negative bacteria, are called endotoxin, and it has been known that lipopolysaccharide is comprised of lipid called lipid A and various kinds of saccharide which covalently bind to the lipid A. It has been also known that this endotoxin has a bioactivity mainly involved in fever, decrease of leukocytes and platelet, hemorrhagic necrosis of bone marrow cells, hypoglycemia, induction of IFN, activation of B limphocyte (immune response cell derived from marrow), and the like.

[0007]

On the other hand, it is known that the function of a specific gene can be analyzed in individual level by using transgenic mice generated by using embryonic stem cells (hereinafter "ES cells"), and knockout mice generated by using gene targeting in which specific genes on genomes are artificially transformed by homologous recombination. In general, gene-deficient mice are called knockout mice, and MyD88 knockout mice have not b n known, and moreover, it has not been known that MyD88 knockout mic are unresponsive to

bacterial cell wall components, either.

[0008]

[An Object to be Attained]

An object of the present invention is to provide MyD88 knockout mice or other non-human animals whose function of MyD88 genes is deficient which can be used for assessing endotoxin activity, IL-1 activity or IL-18 activity of subject materials.

[0009]

[Means to Attain the Object]

The inventors of the present invention have conducted intensive study for attaining the object. They generated MyD88 gene-deficient mice as follows: two exon regions encoding the C-terminal portion of MyD88 gene are replaced with the neomycin-resistant gene by homologous recombination with plasmid vectors in ES cells and HSV-tk gene was induced into C-terminal side, and ES cell clones doubly resistant of G418 and gancyclovir were screened; the ES cell clones were microinjected into blastocysts of C57BL/6 mice; MyD88 knockout mice whose function of MyD88 genes is deficient were born through the germline at the expected Mendelian ratios. Then the inventors have found that those MyD88 knockout mice are transgenic mice which grow healthy and show no obvious abnormalities until 20 weeks of age, and that those MyD88 knockout mice are unresponsive to a cell wall component of Gram-negative bacteria or a cell wall component of Gram-positive bacteria, and the present invention has thus completed.

[0010]

The present invention relates to a bacterial cell wall component-unresponsive non-human animal characterized by that its function of myeloid differentiation primary response gene is deficient on its chromosome (claim 1), the The non-human animal according to claim 1, wherein the bacterial cell wall component is endotoxin derived from Gram-negative bacteria (claim 2), the non-human animal according to claim 1, wherein the bacterial cell wall component is a cell wall component of Gram-positive bacteria (claim 3), the non-human animal according to claim 3, wherein the cell wall component of Gram-positive bacteria is peptidoglycan deived from Gram-positive bacteria, lipoteichoic acid or Mycobacterium tuberculosis lysate (claim 4), the non-human animal according to claim 4, wherein the non-human animal is a rodent (claim 5) and the non-human animal according to claim 5, wherein the rodent is a mouse (claim 6).

[0011]

The present invention also relates to an assessing method of a subject material characterized in comprising the steps of: the subject material is administered to the non-human animal being deficient in function of myeloid differentiation primary response gene: an endotoxin activity of the subject material is assessed (claim 7), an assessing method of a subject material characterized in comprising the steps of: the subject material is administered to the non-human animal being deficient in function of myeloid differ ntiation primary response gene: an

interleukin-1 activity of the subject material is assessed (claim 8), an assessing method of a subject material characterized in comprising the steps of: the subject material is administered to the non-human animal being deficient in function of myeloid differentiation primary response gene; an interleukin-18 activity of the subject material is assessed (claim 9), an assessing method of a subject material characterized in comprising the steps of: the subject material is administered to the non-human animal being deficient in function of myeloid differentiation primary response gene, and a wild-type non-human animal of the non-human animal; an endotoxin activity of the subject material is assessed (claim 10), an assessing method of a subject material characterized in comprising the steps of: the subject material is administered to the non-human animal being deficient in function of myeloid differentiation primary response gene, and a wild-type non-human animal of the non-human animal; an interleukin-1 activity of the subject material is assessed (claim 11), an assessing method of a subject material characterized in comprising the steps of: the subject material is administered to the non-human animal being deficient in function of myeloid differentiation primary response gene, and a wild-type non-human animal of the non-human animal; an interleukin-18 activity of the subject material is assessed (claim 12) and the assessing method of a subject material according to any one of claims 7 to 12, wherein the non-human animal is a mouse (claim 13).

[0012]

The present invention further relates to a knockout mouse characterized in being unresponsive to a bacterial cell wall component (claim 14) and the knockout mouse according to claim 14, wherein the bacterial cell wall component is a cell wall component of Gram-positive bacteria (claim 15).

[0013]

The present invention still further relates to a method of detecting bacterial cell wall components characterized in comprising the steps of: a subject material is administered to a knockout mouse being unresponsive to bacterial cell wall components; bacterial cell wall components in the subject material are detected (claim 16) and a method of detecting bacterial cell wall components characterized in comprising the steps of: a subject material is administered to a knockout mouse being unresponsive to bacterial cell wall components and a wild-type mouse; bacterial cell wall components in the subject material are detected (claim 17).

[0014]

[Mode for Carrying out the Invention]

In the present invention, "deficiency of MyD88 gene function" means that a part of or a whole of MyD88 gene on a chromosome is deficient and the function to express MyD88, which is expressed in wild-types, is lost. Examples of a non-human animal whose function of MyD88 gene is deficient include a rodent such as a rat or the like whose function of MyD88 gene is deficient other than MyD88 knockout mice. As exampl s of the non-human animals whose function of MyD88 gen is deficient,

th ones generated at the expected Mendelian ratio are preferably exemplified considering that wild-type littermates of MyD88-deficient type are obtainable and that precise comparative experiments can be conducted with the animals. With an example of MyD88 knockout mice, a generating method of the non-human animal whose function of MyD88 gene is deficient will now be explained.

[0015]

MyD88 gene can be screened by using gene fractions obtained from a mouse genomic library through PCR. The screened MyD88 gene is subcloned with plasmid vector or the like, and can be characterized by restriction enzyme mapping and DNA sequencing. Then, a targeting vector is replaced with a marker gene such as a neomycin resistance gene or the like, and subsequently the targeting vector is linearized and transfected with embryonic stem cells (ES cells), and then clones, for instance, clones which show resistance to G418 are screened, and obtained targeting ES clones are microinjected into blastocysts of mice. Chimeric mice are mated with female mice, and thus obtained heterozygous mice are intercrossed in order to obtain homozygous mice, then the object MyD88 knockout mice and wild-type mice are generated at the expected Mendelian ratio.

[0016]

It is possible to confirm that the obtained MyD88 knockout mice are unresponsive to a bacterial cell wall component, for example, by injecting LPS, which is a bacterial cell wall

component of Gram-negative bacteria, into MyD88 knockout mice by intravenous injection or the like, and then measuring bioactivity of endotoxin such as fever, shock, decrease of leukocytes or platelet, hemorrhagic necrosis of bone marrow cells, hypoglycemia, induction of IFN, activation of B limphocyte (immune response cell derived from marrow) or the like, or by measuring the induction of IFN, proliferative response of splenic B cells, the expression of MHC class II antigen on the surface of splenic B cells, in macrophages or splenic B cells of MyD88 knockout mice, in the presence of LPS derived from bacteria, or peptidoglycan, which is a cell component of Gram-positive bacteria, lipoteichoic acid, Mycobacterium tuberculosis lysate or the like.

[0017]

The MyD88 knockout mice of the present invention show lower responsiveness to endotoxin than C3H/HeJ mice, which have been known as being hyporesponsive to endotoxin so far, and no shock symptom has been observed. Moreover, macrophages and splenic B cells of MyD88 knockout mice are unresponsive not only to endotoxin but also to peptidoglycan being a cell wall component of Gram-positive bacteria, lipoteichoic acid, Mycobacterium tuberculosis lysate and the like, while they are responsive to IL-4 and IFN-γ. Therefore, the knockout mice being unresponsive to bacterial cell wall components can be used as useful model for elucidating action mechanisms of endotoxin, peptidoglycan, lipoteichoic acid or the like, and for establishing a treatm nt method for endotoxin sh ck.

[0018]

The endotoxin activity of the subject material can be assessed precisely by using the MyD88 knockout mice or the MyD88 knockout mice and wild-type mice, preferably wild-type littermate, as control. By precisely assessing endotoxin activity of a subject material, it becomes possible to obtain useful information for developing antagonists to endotoxin or other such pharmaceuticals which can suppress the shock or fever caused by endotoxin.

[0019]

IL-1 activity of a subject material can be assessed after administering the subject material to the MyD88 knockout mice of the present invention or the MyD88 knockout mice and wild-type mice, preferably wild-type littermate of the MyD88 knockout mice. Examples of IL-1 activity as an object of assessment include mitogens such as phytohemagglutinin (PHA), concanavalin A (Con A) and the like, proliferation inducing activity of T cells caused by co-stimulation with IL-2 at a low concentration, and activity which induces the production of TNF, IL-1 and IL-6 by working on monocytes and macrophages.

[0020]

By using the MyD88 knockout mice or the MyD88 knockout mice and wild-type mice, preferably wild-type littermate, as control, the IL-1 activity of the subject material can be assessed precisely, and the relationship between IL-1 and the illness in disease model mice can be examined. It becomes possible to obtain useful information for developing

pharmaceuticals which can cure diseases such as rheumatoid arthritis caused by overexpression of IL-1, a graft-versus-host disease, asthma and the like by precisely assessing IL-1 activity of a subject material and by analyzing the involvement of IL-1 in disease model mice.

[0021]

IL-18 activity of a subject material can be assessed after administering the subject material to the MyD88 knockout mice of the present invention or the MyD88 knockout mice and wild-type mice, preferably wild-type littermate of the MyD88 knockout mice. Examples of IL-18 activity as an object of assessment include activity which promotes production of IFN-γ, activity which enhances activity of NK cells, activity which induces production of IFN-γ from T cells in cooperation with IL-12, and action which activates IRAK or $NF_{\kappa}B$.

[0022]

By using the MyD88 knockout mice or the MyD88 knockout mice and wild-type mice, preferably wild-type littermate, as control, the IL-18 activity of the subject material can be assessed precisely. It becomes possible to obtain useful information for developing pharmaceuticals which can cure diseases caused by overproduction of IL-18, such as I type diabetes, a graft-versus-host disease and the like by precisely assessing IL-18 activity of a subject material.

[0023]

[Examples]

The present invention will be explained more specifically

with examples below, but the technological scope of the present invention is not limited to these examples.

Example 1 (Generation of MyD88 knockout mice)

A MyD88 gene was screened from a 129/SvJ mouse genomic library (Stratagene), subcloned into pBluescript vector (Stratagene), and characterized by restriction enzyme mapping and DNA sequencing. A targeting vector was constructed by replacing the 1.0 kb genomic fragment with a neomycin resistance gene from pMC1-neo (Stratagene). The replaced genomic fragment contained 2 exons encoding the domain that resembles the cytoplasmic domain of the IL-1RAcP (receptor accessory protein). The neomycin resistance gene was flanked by the 1.1 kb 5' genomic fragment and the 5.2 kb 3' fragment. Then, an HSV-tk cassette was introduced into the 3' end of the genomic fragment. E14.1 ES cells were transfected with the linearized targeting vector and selected with G418 and gancyclovir. Doubly resistant 176 clones were screened for homologous recombination by PCR and 33 clones were verified by Southern blot analysis using the probe indicated in Fig. 1.

[0024]

Three independently identified targeted ES clones were microinjected into the blastocysts of C57BL/6 mice. Thus obtained chimeric mice were mated with C57BL/6 female mice to produce heterozygous mice. The Heterozygous mice were intercrossed to obtain homozygotes, and MyD88-deficient were born at the xpect d M ndelian ratios (+/+:+/-:-/- = 52:93:53) from the intercross. The MyD88 knockout mice of the present

invention gr w healthy and showed no obvious abnormalities until 20 weeks of age. Northern blot analysis was performed to confirm that the inactivation of the MyD88 gene was caused by mutation. MyD88 mRNA could not be detected in the liver and the spleen of the MyD88-deficient mice. Flow cytometric analysis of CD3, B220, CD4, and CD8 in thymus, spleen, and lymph node showed that lymphocyte composition was not altered in the MyD88 knockout mice in comparison with wild-type mice.

[0025]

Example 2 (Unresponsiveness of MyD88 knockout mice to Endotoxin)

1mg of LPS derived from Escherichia coli (O55:B5) was administered to 10 MyD88 knockout mice of the present invention, and endotoxin-unresponsiveness was examined through the survival ratio of the mice. 10 wild-type littermates were used as control. The results are shown in Fig. 2. It is confirmed by Fig. 2 that though the wild-type mice have responded to LPS and all of them died within 4 days after administration, none of the MyD88 knockout mice of the present invention have died within 4 days after LPS administration, and that the mice are endotoxin-unresponsive.

[0026]

Example 3 (Impaired IL-1-mediated functions in MyD88 knockout mice)

1×10^5 of thymocytes of the MyD88 knockout mice of the present invention were cultured in 96-well plates for 72 hours with mixtures containing 2 μg/ml of phytohemagglutinin (PHA),

which is a costimulant of IL-1 for T c ll proliferation, 2.5 μg/ml of concanavalin A (ConA), 2 ng/ml of IL-2 respectively, and 100 U/ml of IL-1β (Genzyme), and T cells were proliferated. Proliferation of T cells were examined by measuring [^{3}H] amount of [^{3}H] thymidine taken into the cells. As a result, thymocytes of wild-type littermates displayed enhanced proliferation when cultured with PHA, ConA or IL-2 in the presence of IL-β, however, thymocytes of the MyD88 knockout mice of the present invention show almost no enhanced proliferation (see Fig. 3). It has been found that similar results could be obtained even when splenic B cells were used instead of thymocytes.

[0027]

Further, thymocytes of MyD88 knockout mice of the present invention were cultured with 10 ng/ml of phorbol 12-myristate 13-acetate paramethoxyamphetamine (PMA) or 2.5 μg/ml of Con A in the presence of 20 ng/ml of IL-2 (Genzyme) in a same manner as above-mentioned, and enhancement of proliferation was examined. There was no difference between thymocytes of MyD88 knockout mice of the present invention and of wild-type littermates in their proliferation as to the reaction of IL-2 and PMA or Con A (see Fig. 3). These results indicate that IL-1-mediated growth signal of T cells was impaired in the thymocytes of MyD88 knockout mice of the present invention.

[0028]

MyD88 knockout mice of the present invention were intravenously injected with 1 μg of IL-β (Genzyme), and 2 hours later liver and sera wer taken. Total RNA was xtracted from

th liver using Trizol reagent (GIBCO). This RNA (10μg) was electrophoresed and transferred to a nylon membrane, then Northern blot analysis was conducted with ^{32}P-labelled cDNA for acute phase proteins such as serum amyloid A (SAA-I), serum amyloid P(SAP), and haptoglobin (HP). In comparing IL-1-induced increase of mRNA expression in wild-type littermates and in MyD88 knockout mice of the present invention, increase of induction was observed in wild-type mice, but not observed in MyD88 knockout mice.

[0029]

Because IL-1 induces production of acute phase proteins such as tumor necrosis factor (TNF) or IL-6, and proinflammatory cytokines, increase of TNF and IL-6 concentrations in serum taken from MyD88 knockout mice of the present invention and wild-type littermates by the above-stated method were measured by ELISA. As a result, TNF and IL-6 concentrations increased by IL-1β in wild-type mice, while neither TNF nor IL-6 concentration increased by IL-1β in MyD88 knockout mice (see Fig.4).

Thus, IL-1-mediated major biological functions has been found to be severely impaired in MyD88 knockout mice of the present invention.

[0030]

Example 4 (Impaired IL-18-mediated functions in MyD88 knockout mice)

It has been well known that IL-18 enhances lytic activity of NK cells. Splenocytes from MyD88 knockout mice of the

present inv ntion and wild-type littermates were cultured in the presence or absence of 20 ng/ml of IL-18 (Hayashibara Biochemical Laboratories, Inc.) for 24 hours with ^{51}Cr-labelled mouse lymphoma cells (hereinafter "YAC-1") targeting cells. 4 hours later, released ^{51}Cr in supernatants were counted by a gamma counter. As a result, when splenocytes were cultured in the presence of IL-18 in vitro, lytic activity to YAC-1 targeting cells was dramatically enhanced in wild-type mice, but it was not enhanced in MyD88 knockout mice. When IL-2 was used instead of IL-18, lytic activity was also enhanced in splenic B cells of MyD88 knockout mice of the present invention (see Fig.5).

[0031]

Further, splenic B cells of MyD88 knockout mice of the present invention and their wild-type littermates were stimulated by 20 ng/ml of IL-18 and cultured for 24 hours in vitro, then production of IFN-γ in culture supernatants was measured by ELISA. As a result, production of IFN-γ was induced in wild-type mice, however, production of IFN-γ was not observed in MyD88 knockout mice of the present invention (see Fig.5).

[0032]

Splenic T cells of MyD88 knockout mice of the present invention and their wild-type littermates, which were purified to 95% or over, were cultured on anti-CD3 antibody (20 μg/ml)(PharMingen)-coated plat s in the presence of 2 ng/ml IL-12. 4 days aft r the onset of culture, cells w re harvested

and wash d with Hanks' balanced salt solution. The washed cells (2×10^5) were stimulated and cultured again on anti-CD3 antibody (20 μg/ml)-coated 96-well plates for 24 hours with 20 ng/ml of IL-18 or 2 ng/ml of IL-12. Concentration of IFN-γ in culture supernatants was determined by ELISA and compared. The result indicates that Splenic T cells of MyD88 knockout mice of the present invention cannot enhance IL-18-responsive production of IFN-γ (see Fig.6).

[0033]

MyD88 knockout mice of the present invention and their wild-type littermates were intraperitoneally injected with 500 μg of heat-killed Propionibacterium acnes (P. acnes). Seven days after injection, T cells were purified from spleen, then cultured and stimulated on anti-CD3 antibody (20 μg/ml)-coated 96-well plates for 24 hours in the presence or the absence of 20 ng/ml of IL-18. Concentration of IFN-γ in culture supernatants was determined by ELISA. MyD88 knockout mice of the present invention and their wild-type littermates were intravenously injected with 2 mg of Bacillus Calmette-Guérin (BCG) (Kyowa). 14 days after injection, T cells were purified from spleen, then cultured and stimulated for 24 hours, as described above, subsequently concentration of IFN-γ was measured. As a result, in both cases, high level of IFN-γ production in response to IL-18 was observed in wild-type mice, but production level of IFN-γ could not be enhanced in the presence of IL-18 in MyD88 knockout mic of the present invention (see Fig.6).

[0034]

These results demonstrate that MyD88 knockout mice of the present invention are defective in Th1 cell development in vivo as is the case with IL-18-deficient mice, and that their major biological activities mediated by IL-18 were completely abolished.

[0035]

Next, it was examined whether the dominant negative MyD88 mutant blocked IL-18-induced NF-$_\kappa$B activation as well. COS-7 cells were transiently transfected with MyD88 (amino acid 152-296) expression vector together with NF-$_\kappa$B-dependent luciferase reporter gene, and luciferase activity after IL-18 treatment was measured. Coexpression of MyD88 blocked IL-18-induced activation almost completely (see Fig. 7).

[0036]

Because IL-18 activates AP-1-dependent gene information, whether MyD88 (amino acid 152-296) also acted as a dominant negative mutant of IL-18-induced AP-1 activation was investigated. Stimulation with IL-18 induced an approximately 3- to 4-fold increase in AP-1 activity, and this activation was blocked by coexpression of MyD88 (amino acid 152-296) (see Fig. 7). These results show that MyD88 is involved in IL-18-induced activation of both NF-$_\kappa$B and AP-1.

[0037]

Further, whether IL-18-induced activation of NF-$_\kappa$B was observ d in MyD88-deficient cells was examined. Splenic T cells cultured in the presence of IL-12 and anti-CD3 antibody

for 4 days were starved for 3 hours and then stimulat d with IL-18. Nuclei extracted from the stimulated cells were analyzed by a gel mobility shift assay using a specific probe containing NF-$_\kappa$B binding site. IL-18-induced NF-$_\kappa$B DNA binding activity was detected in the nuclear extracts from wild-type cells but not from MyD88-deficient cells. On the other hand, treatment of wild-type or MyD88-deficient thymocytes with TNFα resulted in almost the same levels of NF-$_\kappa$B DNA binding activity, demonstrating that the impaired IL-18-induced NF-$_\kappa$B activity in MyD88-deficient cells was not due to the abnormal function or impairment of regulating ability of NF-$_\kappa$B.

[0038]

In addition to induction of NF-$_\kappa$B activation, IL-1 is also known to activate c-Jun N-terminal kinase (JNK). To test whether IL-18 induces JNK activation, an in vitro kinase assay was carried out using GST-c-Jun-fusion protein as a substitute. Treatment with IL-18 induced JNK activation in Th1-developing cells of wild-type mice. However, IL-18-induced JNK activation was not observed in MyD88-deficient cells. By contrast, normal activation of JNK was observed in MyD88-deficient cells treated with TNF-α. IL-18-induced NF-$_\kappa$B and JNK activation was impaired in MyD88-deficient mice. These results demonstrate that MyD88 is essential for IL-18-induced activation of both NF-$_\kappa$B and JNK.

[0039]

Example 5 (Unresponsiveness of macrophages and spl nic B cells

of MyD88 knockout mice to bacterial cell wall components)

5-1 (Generation of TLR4-deficienct mice)

It has recently been reported that C3H/HeJ mice are hyporesponsive to LPS because of a missense point mutation in the Toll-like receptor(TLR)-4 gene (Science 282, 2085-8, 1998, J. Exp. Med. 189, 615-625, 1999, J. Immunol. 162, 3749-3752, 1999), and the inventors have demonstrated that macrophages and splenic B cells of TLR4-deficient mice are hyporesponsive to LPS, and that TLR4 gene is essential for LPS signaling (J. Immunol. 162, 3749-3752, 1999). In order to compare the responsiveness of macrophages and splenic B cells of TLR4- and MyD88-deficient mice to bacterial cell wall components, TLR4-deficient mice (F_2 interbred from 129/OlaXC57BL/6) were generated by gene targeting as described previously (J. Immunol. 162, 3749-3752, 1999). Age-matched groups of wild-type, TLR4-, and MyD88-deficient mice were used for the following examples.

[0040]

5-2 (Preparation of bacterial cell wall components)

LPS of Escherichia coli Serotype 055:B5 (Sigma), Klebsiella pneumoniae (Sigma), Pseudomonas aeruginosa Serotype 10 (Sigma), Salmonella typhimurium (Sigma), Serratia marcescens (Sigma), Shigella flexneri Serotype 1A (Sigma) and Vibrio cholerae Serotype Inaba 569B (Sigma) and the like were purchased. They were prepared by phenol extraction and purified by gel filtration. LPS from Salmonella minnesota Re-595 prepared by ph nol-chloroform-petroleum ether extraction proc dure was also purchased (Sigma). LPS and Lipid

A of Porphyromonas gingivalis 381 was prepared by the method as described previously (FEBS Lett. 332, 197-201, 1994). Whole cell lysates of Mycobacterium tuberculosis was prepared by the following process: Mycobacterium tuberculosis Aoyama B strain (NIHJ 1635) was cultured in Dubos broth (DIFCO) for 1 month; cells were collected and resuspended with phosphate buffered saline (PBS); cells were sonicated.

[0041]

5-3 (Preparation of peritoneal macrophages)

2 ml of 4% thioglycollate was intraperitoneally injected into the generated wild-type, TLR4- and MyD88-deficient mice respectively. Three days later, peritoneal exudate cells were isolated from the peritoneal cavity and washed with ice-cold Hank's buffered salt solution (HBSS), then peritoneal cells were obtained. The cells were made to float in RPMI1640 medium, then put in plastic plates separatedly, and cultured for 2 hours at 37° C and washed with Hank's buffered salt solution to remove nonadherent cells. Adherent cells were used as peritoneal macrophages in the experiments bellow.

[0042]

5-4 (Unresponsiveness to LPS of Salmonella minnesota Re-595)

Responsiveness of each peritoneal macrophage of the wild-type, TLR4-deficient, MyD88-deficient mice and the like to LPS were examined with LPS of Salmonella minnesota Re-595. Peritoneal macrophages from each mouse were cultured for 24 hours in the presence of various conc ntrations (0, 0.01, 0.1, 1, 10 or 100 μg/ml) of LPS and stimulated, then concentration

of tumor necrosis factor (TNF-α) released from LPS-responsive macrophages was measured by ELISA (see Fig. 8A). By these results, it has been found that production of TNF-α increases in response to LPS in a dose-dependent manner in macrophages of wild-type mice, by contrast, no production of TNF-α is observed in TLR4- or MyD88-deficient mice even when they receive LPS stimuli at a concentration of 100 μg/ml, and that these mice are LPS-unresponsive.

[0043]

Further, responsiveness of splenic B cells to LPS of Salmonella minnesota Re-595 was examined. Splenic B cells (1×10^5) of each of the wild-type, TLR4- and MyD88-deficient mice were isolated, cultured in 96-well plates and stimulated by various concentrations (0, 0.01, 0.1, 1, 10 or 100 μg/ml) of LPS. 1 μCi of [^{3}H]-thymidine (DuPont) was added 40 hours after onset of the culture, then the cells were cultured for another 8 hours, and [^{3}H] uptake was measured by a β scintillation counter (Packard) (see Fig. 8B). As a result, LPS-induced proliferative response was promoted in response to LPS in a dose-dependent manner in splenic B cells of wild-type mice, by contrast, no LPS-induced proliferative response was observed in splenic B cells of both TLR4- and MyD88-deficient mice.

[0044]

The expression of major histocompatibility complex (MHC) class II (I-A molecule) on the surface of splenic B cells in response to R -595 LPS was examined by flow cytometry. Splenic B cells (1×10^6) from each of the wild-type, MyD88- and

TLR4-deficient mic were cultured for 48 hours in the pr sence of various concentrations (0, 0.01, 0.1, 1, 10 or 100 μg/ml) of LPS. After the culture, the cells were collected and then stained by combining I-A^b molecule on the surface of the cells and FITC-labelled antibody which is constructed by combining phycoerythrin (PE; PharMingen)-conjugated anti-B220 antibody or biotinylated anti-mouse I-A antibody (PharMingen) and fluorescein isocyanate (FITC; PharMingen)-conjugated streptavidin. The stained cells were analyzed on fluorescence-activated cell sorter Calibur (FACS Calibur) using CELLQuest software (Becton Dickinson). As a result, Re-595 LPS caused an increase in the expression of I-A molecule on the surface of splenic B cells of wild-types. In contrast, Re-595 LPS did not enhance I-A molecule expression in splenic B cells of either TLR4- or MyD88-deficient mice, even when stimulated with high concentration of LPS (100 μg/ml)(see Fig. 8C). The above-mentioned results indicate that both TLR4- and MyD88-deficient mice are unresponsive to LPS of Salmonella minnesota Re-595.

[0045]

5-5 (Responsiveness of TLR4- and MyD88-deficient mice to IL-4 and IFN-γ)

In order to examine whether splenic B cells of TLR4- and MyD88-deficient mice are unresponsive to all stimuli, the responsiveness of splenic B cells of TLR4- and MyD88-deficient mice to other stimuli w re investigated. The investigation demonstrates that there was no impairment as to their

responsiveness to the stimuli as described below, and that these mice were specifically defective in their response to LPS.

[0046]

Splenic B cells (1×10^5) from each of the wild-type, MyD88- and TLR4-deficient mice were isolated, cultured for 40 hours in the presence of both IL-4 (Genzyme) and anti-IgM antibody, or in the presence of anti-CD40 antibody, then [^{3}H]-thymidine (DuPont) was added and the cells were cultured for another 8 hours, and [^{3}H] uptake was measured by a β scintillation counter (see Fig. 9A). As a result, splenic B cells of both TLR4- and MyD88-deficient mice showed same reaction as splenic B cells of wild-type mice with regard to the response to IL-4 and the mixture of anti-IgM antibody, or to the anti-CD40 antibody.

[0047]

Next, Splenic B cells (1×10^6) from each of the wild-type, MyD88- and TLR4-deficient mice were cultured for 48 hours in the presence or absence of 100 U/ml of IL-4, and then stimulated. Subsequently, the cells were stained by combining I-A molecule on the surface of the splenic B cells and PE-conjugated anti-B220 antibody or FITC-conjugated anti-mouse I-A^b antibody. The cell proliferation was measured on fluorescence-activated cell sorter Calibur using CELLQuest software (see Fig. 9B). As a result, splenic B cells of both TLR4- and MyD88-deficient mice showed same reaction as those of wild-type mice with regard to the response to IL-4 as well.

[0048]

Each of wild-type, MyD88- and TLR4-deficient mice were intraperitoneally injected with 5000 U of IFN-γ (Genzyme) or PBS. Three days after injection, peritoneal macrophages were collected and stained by combining I-A molecule on the surface of the macrophage membranes and FITC-conjugated anti-mouse $I\text{-}A^b$ antibody, then analyzed by fluorescence-activated cell sorter Calibur using CELLQuest software (see Fig. 9C). The result indicated that the expression of I-A molecule in peritoneal macrophages, in other words, blockage level of IFN-γ-induced cell proliferation was comparative among wild-type, MyD88- and TLR4-deficient mice.

[0049]

5-6 (Analysis of phagocytosis)

Macrophages of wild-type, MyD88- and TLR4-deficient mice added with 0.025 % of fluorescent latex beads (0.75 μm) (Polyscience) were cultured for 2 hours at 37°C in a CO_2 incubater. Then the culture materials were washed vigorously three times with PBS to remove non-phagocytosed beads and incubated with PBS containing 2.5 % of formaldehyde for 20 minutes, and fixed with formaldehyde. Visualization of these fixed cells with Axiophoto microscope (Carl Zeiss, Inc.) showed that peritoneal macrophages of both TLR4- and MyD88-deficient mice phagocytosed the latex particles, and therefore, that phagocytic ability of the macrophages of TLR4- and MyD88-deficient mice were not impaired by these other stimuli.

[0050]

5-7 (Responsiveness to LPS of Porphyromonas gingivalis)

As LPS of Porphyromonas gingivalis shows some reaction in its ability to activate cells of LPS-hyporesponsive C3H/HeJ mice (J. Immunol. 158, 4430-6, 1997), responsiveness of each mouse to LPS of Porphyromonas gingivalis was examined as in the case with Salmonella minnesota Re-595. In macrophages of wild-type mice, TNF-α was induced in response to LPS of Porphyromonas gingivalis in a dose-dependent manner. However, macrophages of TLR4-deficient mice were hyporesponsive like those of C3H/HeJ mice, and only showed the TNF-α producibility which was about one third of that of wild-type mice macrophages. In contrast, macrophages of MyD88-deficient mice did not produce any detectable TNF-α, even when stimulated with high concentration of LPS (see Fig. 10A).

[0051]

Splenic B cells of TLR4-deficient mice exhibited low level proliferative response to LPS of Porphyromonas gingivalis 381, and enhanced the expression of I-A molecule of splenic B cells, however, splenic B cells of MyD88-deficient mice did not exhibit proliferative response and the expression of I-A molecule could not confirmed (see Fig. 10B and C). Further, the same results were obtained with lipid A of Porphyromonas gingivalis 381. This indicates that TLR4-deficient mice are hyporesponsive and MyD88-deficient mice are unresponsive to LPS of Porphyromonas gingivalis. In addition, it has been found that MyD88 is essential for the signaling induced by LPS of Porphyromonas gingivalis, whereas TLR4 shows partial contribution.

[0052]

5-8 (Responsiveness to LPS of Escherichia coli O55:B5)

Responsiveness to LPS of Escherichia coli O55:B5 was examined as in the case with Salmonella minnesota Re-595. The responsiveness to LPS of Escherichia coli (O55:B5) was impaired in peritoneal macrophages of both TLR4- and MyD88-deficient mice, compared with those of wild-type mice (Fig. 11A). However, when stimulated with high concentration of LPS, macrophages of TLR4-deficient mice produced a small amount of TNF-α. In contrast, macrophages of MyD88-deficient mice did not produce TNF-α even when stimulated with high concentration of LPS.

[0053]

Similar tendencies were observed in proliferative responses in splenic B cells of these mice (see Fig. 11B). Furthermore, when stimulated with LPS at a concentration over 10 μg/ml, splenic B cells of TLR4-deficient mice showed a certain expression level of I-A molecule similar to the level shown by splenic B cells of wild-type mice. In contrast, splenic B cells of MyD88-deficient mice did not show I-A molecule expression even when stimulated with LPS at a concentration of 100 μg/ml (see Fig. 11C). As in the case of stimuli with LPS of Porphyromonas gingivalis, these results indicate that TLR4-deficient mice are hyporesponsive and MyD88-deficient mice are unresponsive to LPS of Escherichia coli (O55:B5).

[0054]

Example 10 (Responsiveness to peptidoglycan)

It has been reported that Peptidoglycan (PGN), which is a major cell wall component of Gram-positive bacteria, activates macrophages (J. Immunol. 155, 2620-30, 1995, Infect. Immun. 62, 2715-21, 1994). Therefore, responsiveness to PGN of Staphylococcus aureus (Fluka) was examined as in the case with Salmonella minnesota Re-595. When stimulated with PGN, peritoneal macrophages of TLR4-deficient mice produced TNF-α in a dose-dependent manner to almost the same extent as macrophages of wild-type mice. In contrast, macrophages of MyD88-deficient mice did not produce TNF-α even when stimulated with high concentration of PGN (see Fig. 12A).

[0055]

When stimulated with PGN of Staphylococcus aureus, splenic B cells of wild-type mice displayed proliferative responses, and the proliferative response was severely impaired in peritoneal macrophages of MyD88-deficient mice compared with those of wild-type mice, but in TLR4-deficient mice, the proliferative response was not severely impaired as in MyD88-deficient mice (see Fig. 12B). Further, when stimulated with PGN at a concentration over 10 μg/ml, splenic B cells of TLR4-deficient and wild-type mice showed enhancement of I-A molecule expression. In contrast, splenic B cells of MyD88-deficient mice did not show enhancement of I-A molecule expression even when stimulated with PGN at a concentration of 100 μg/ml (see Fig. 12C). Thus, TLR4-deficient mice showed almost the sam r sponse to PGN of Staphylococcus aureus as

wild-type mice, while MyD88-deficient mice showed no responsiveness.

[0056]

5-10 (Responsiveness to lipoteichoic acid)

As lipoteichoic acid (LTA) is a cell wall component of Gram-positive bacteria and induces activation of monocytes and macrophages (Infect. Immun. 62, 2715-21, 1994), responsiveness to LTA of Streptococcus pneumoniae was examined as in the case with Salmonella minnesota Re-595. Peritoneal macrophages of wild-type mice increased production of TNF-α in response to LTA in a dose-dependent manner. In contrast, macrophages of MyD88-deficient mice did not produce TNF-α even when stimulated with high concentration of LTA. In comparison with peritoneal macrophages of wild-type mice, TNF-α production was also impaired in those of TLR4-deficient mice, however, TNF-α was induced when stimulated with 100 μg/ml of LTA (see Fig. 13A).

[0057]

Next, proliferative responses and enhancement of I-A molecule expression in splenic B cells of these mice in response to stimulation from LTA of Streptococcus pneumoniae was analyzed (see Fig. 13B). The results indicated that splenic B cells of wild-type mice enhanced their response to LTA in a dose-dependent manner, whereas splenic B cells of MyD88-deficient mice exhibited a severely defective proliferative response to LTA. Though splenic B cells of TLR4-d ficient mice also exhibited an impaired proliferative response, they

xhibited proliferative response when stimulated with high concentration of LTA. Further, in splenic B cells of wild-type and TLR4-deficient mice, enhancement of I-A molecule expression was also observed on the cell surface, whereas no enhancement was observed in those of MyD88-deficient mice (see Fig. 13C). This indicates that MyD88-deficient mice are unresponsive to stimulation from LTA of Streptococcus pneumoniae.

[0058]

5-11 (Responsiveness to whole cell lysates of Mycobacterial tuberculosis)

As cell wall components of Mycobacterial tuberculosis, especially lipoarabinomannan, are known to induce activation of myeloid cells (J. Immunol. 149, 541-7, 1992, J. Clin. Invest. 91, 2076-83, 1993), responsiveness to whole cell lysates of Mycobacterial tuberculosis was examined as in the case with Salmonella minnesota Re-595. Macrophages of wild-type mice produced TNF-α in response to the whole lysates in a dose-dependent manner. Macrophages of TLR4-deficient mice also exhibited TNF-α production though the production amount was smaller than those of wild-type mice. However, macrophages of MyD88-deficient mice did not produce TNF-α in response to the whole cell lysates of Mycobacterial tuberculosis at a high concentration (see Fig. 14A).

[0059]

Next, responsiv ness of these mice to stimulation from whole c ll lysates of Mycobacterial tuberculosis was examined.

Splenic B cells of wild-type mice exhibited enhancement of proliferative responses and I-A molecule expression on the surface of the cells in response to the whole cell lysates in a dose-dependent manner. Splenic B cells of TLR4-deficient mice also showed proliferative responses and I-A molecule expression, although these responses were lower than those of splenic B cells of wild-type mice. In contrast, splenic B cells of MyD88-deficient mice displayed severely impaired proliferative responses and enhancement of I-A molecule expression, indicating that they are unresponsive to the whole cell lysates (see Fig. 14B and C).

[0060]

5-12 (Responsiveness to other bacterial cell wall components)

Responsiveness of wild-type, TLR4- and MyD88-deficient mice to other bacterial cell wall components [LPSs of Klebsiella pneumoniae, Pseudomonas aeruginosa 10, Salmonella typhimurium, Shigella flexneri, Vibrio cholerae and the like, and PGN of Staphylococcus epidermidis, which is provided from Shigeo Kawata of Dainippon Pharmaceutical Co.] was examined in a same manner as aforementioned. The results are shown in Table 1. This Table 1 shows that MyD88-deficient mice are unresponsive to all bacterial cell components.

[0061]

[Table 1]

Test Specimen	The responsiveness of mice		
LPS	wild type	TLR4-/-	MyD88-/-
Escherichia coli O55:B5	++	+	-
Klebsiella pneumoniae	++	-	-
Porphyromonas gingivalis	++	+	-
Pseudomonas aeruginosa	++	+	-
Salmonella minnesota Re595	++	-	-
Salmonella typhimurium	++	+	-
Serratia marcescens	++	+	-
Shigella flexneri	++	+	-
Vibrio cholerae	++	+	-
PGN			
Staphylococcus aureus	++	++	-
Staphylococcus epidermidis	++	+	-
LTA			
Streptococcus faecalis	++	+	-
Mycobacterial whole cell lysates			
Mycobacterium tuberculosis	++	+	-

[0062]

It has been found that LPS can be classified into two types: one type includes LPSs which utilize TLR4 as their unique signaling receptor and show unresponsiveness (LPSs of Salmonella minnesota Re595, Klebsiella pneumoniae and the

like); another type includes LPSs which show hyporesponsiveness to TLR4-deficient mice (LPSs of Porphyromonas gingivalis, Escherichia coli O55:B5, Pseudomonas aeruginosa, Shigella flexneri, Salmonella typhimurium, Vibrio cholerae and the like). Since MyD88-deficient mice show no responsiveness to these latter LPSs, it is presumed that the recognition and signaling of these LPSs are mediated by both TLR4 and other TLRs, and/or by TLR-related receptors that use MyD88 as an adaptor molecule.

[0063]

[Effect of the Invention]

The MyD88 knockout mouse of the present invention is unresponsive to a bacterial cell wall component and its biological function mediated by IL-1 and IL-18 is deficient. Therefore, by using the MyD88 knockout mouse of the present invention, it becomes possible to assess endotoxin activity, IL-1 activity and IL-18 activity in subject materials, and to obtain useful information for development of medicines for diseases caused by excessive production of endotoxin or other bacterial cell wall components, IL-1, IL-18 or receptors of these materials.

[Brief Description of Drawings]

[Fig. 1]

This is a graph showing gene maps of the MyD88 knockout mice and the wild-type mice of the present invention.

[Fig. 2]

This is a graph showing survival indices of the MyD88 knockout mice and the wild-type mice of the present invention

having an injection of LPS d rived from Escherichia coli.

[Fig. 3]

This is a graph showing the results of T cell proliferation mediated by IL-1 in the MyD88 knockout mice and the wild-type mice of the present invention.

[Fig. 4]

This is a graph showing the results of IL-1-induced TNF and IL-6 levels in blood in the MyD88 knockout mice and the wild-type mice of the present invention.

[Fig. 5]

This is a graph showing the results of NK cell activation mediated by IL-18 in the MyD88 knockout mice and the wild-type mice of the present invention.

[Fig. 6]

This is a graph showing the results of the production of IFN-γ stimulated by IL-12 and IL-18 in the MyD88 knockout mice and the wild-type mice of the present invention.

[Fig. 7]

This is a graph showing that the mutation of dominant negative MyD88 is involved in IL-18-induced NF-$_{\kappa}$B activity and AP-1 activity.

[Fig. 8]

This is a graph showing the results of responsiveness of macrophages and splenic B cells of the MyD88 knockout mice, the wild-type mice and the TLR4 knockout mice of the present invention to Salmonella minnesota Re-595.

[Fig. 9]

This is a graph showing the results of responsiveness of macrophages and splenic B cells of the MyD88 knockout mice, the wild-type mice and the TLR4 knockout mice of the present invention to IL-4 and interferon-γ.

[Fig. 10]

This is a graph showing the results of responsiveness of macrophages and splenic B cells of the MyD88 knockout mice, the wild-type mice and the TLR4 knockout mice of the present invention to Porphyromonas gingivalis.

[Fig. 11]

This is a graph showing the results of responsiveness of macrophages and splenic B cells of the MyD88 knockout mice, the wild-type mice and the TLR4 knockout mice of the present invention to Escherichia coli O55:B5.

[Fig. 12]

This is a graph showing the results of responsiveness of macrophages and splenic B cells of the MyD88 knockout mice, the wild-type mice and the TLR4 knockout mice of the present invention to peptidoglycan.

[Fig. 13]

This is a graph showing the results of responsiveness of macrophages and splenic B cells of the MyD88 knockout mice, the wild-type mice and the TLR4 knockout mice of the present invention to lipoteichoic acid.

[Fig. 14]

This is a graph showing the results of responsiveness of macrophages and splenic B cells of the MyD88 knockout mice, the

wild-typ mice and the TLR4 knockout mice of the present invention to whole cell lysates of Mycobacterium tuberculosis.

[Name of Docum nt] Abstract

[Abstract]

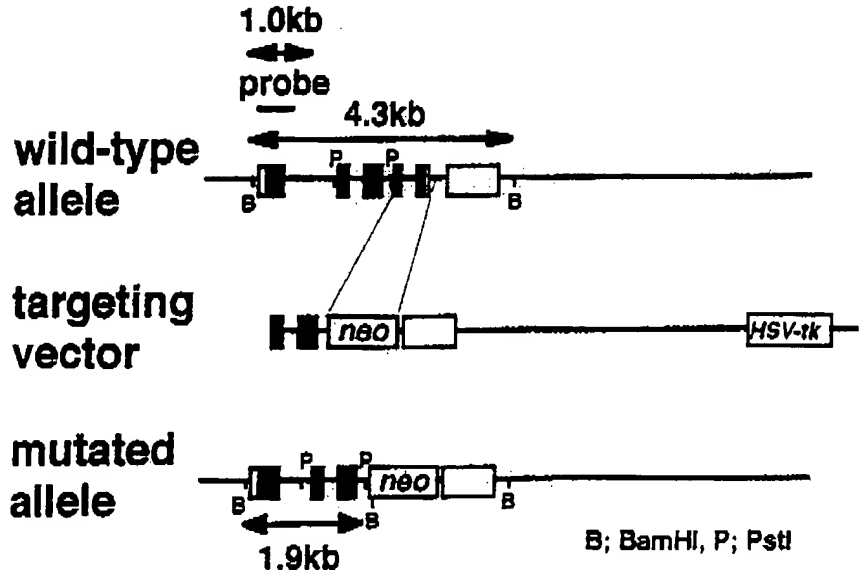
[The Object] The object of the present invention is to provide non-human animals whose function of myeloid differentiation primary response (MyD88) genes is deficient such as knockout mice whose function of myeloid differentiation primary response (MyD88) genes is deficient, and knockout mice being unresponsive to bacterial cell wall components such as endotoxin, peptidoglycan or the like, which can be used for assessing endotoxin activity, IL-1 activity or IL-18 activity of subject materials.

[Solving Means] Generating knockout mice being unresponsive to bacterial cell wall components which can be used for assessing endotoxin activity, IL-1 activity or IL-18 activity of the subject material after administering the subject material to knockout mice whose function of MyD88 genes is deficient and to wild-type mice.

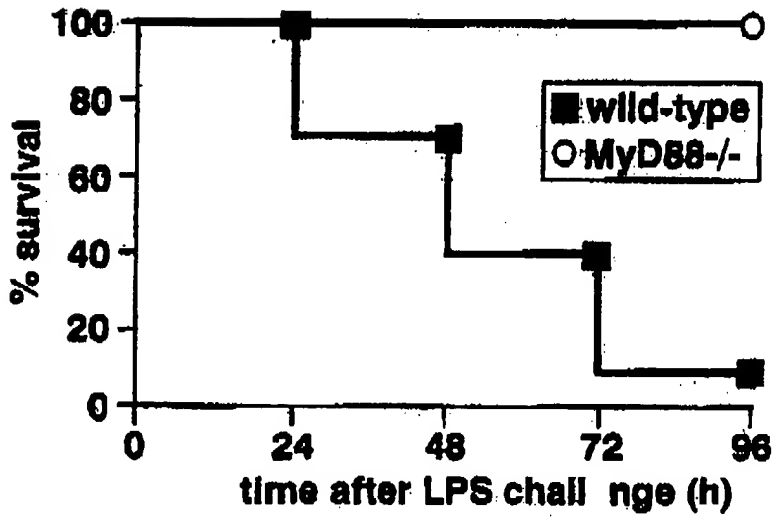
[Selected Drawing] Fig. 1

[Name of Document] Drawing

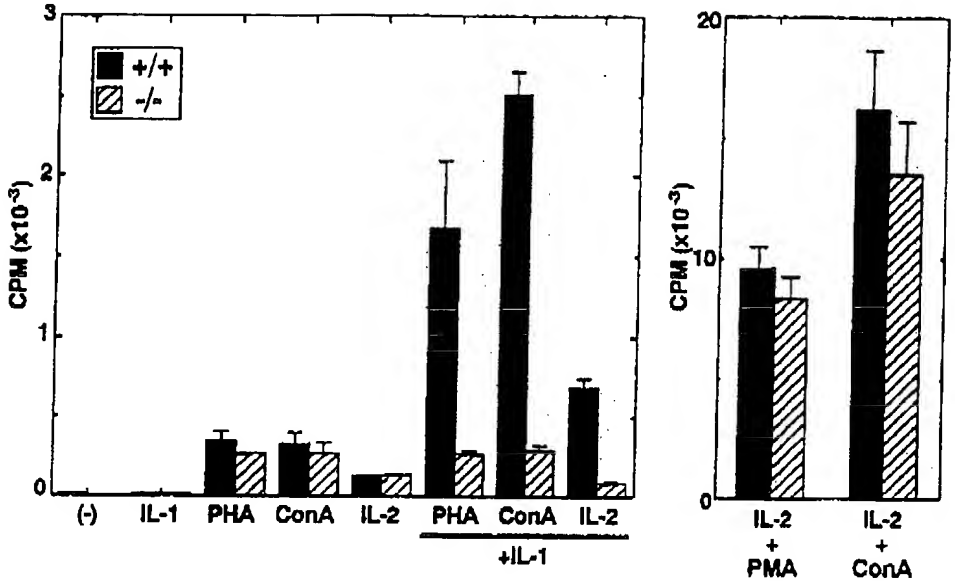
[Fig. 1]



[Fig. 2]



[Fig. 3]


+/+
-/-
CPM (x10⁻³)
3
2
1
0
(-)
IL-1
PHA
ConA
IL-2
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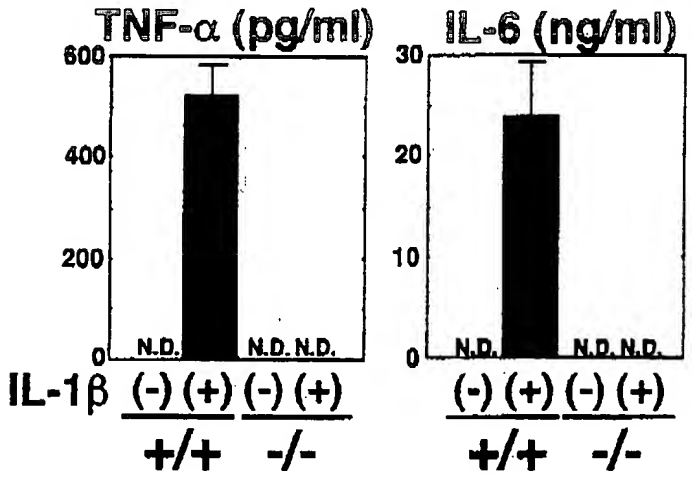
ConA
IL-2
+IL-1
20
10
0
IL-2 + PMA
IL-2 + ConA

[Fig. 4]


TNF-α (pg/ml)
IL-6 (ng/ml)
600
400
200
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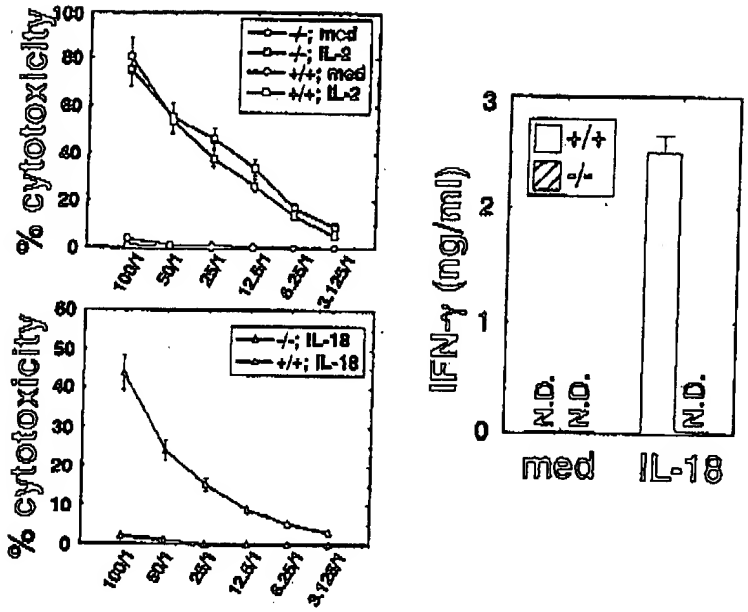
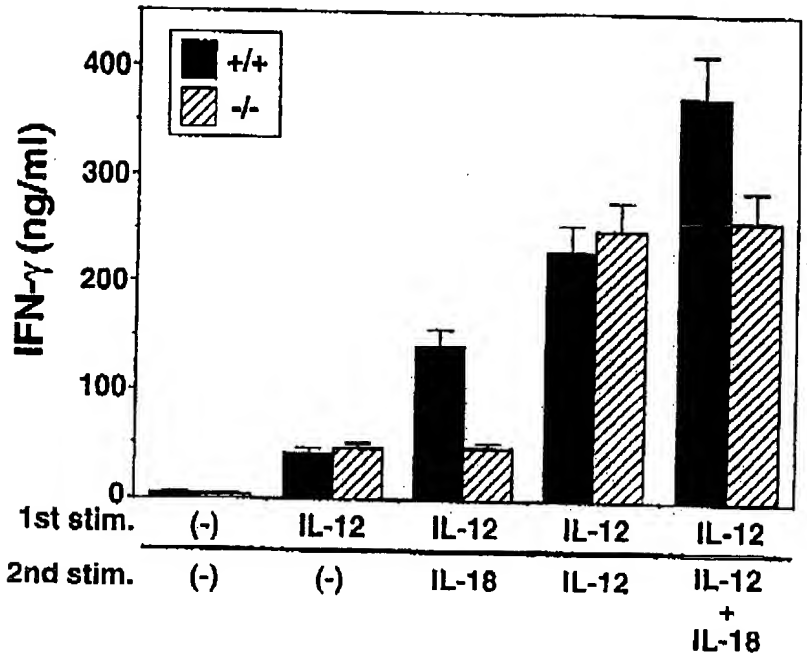
20
10
0
N.D.
N.D. N.D.
IL-1β (-) (+) (-) (+)
+/+ -/-

[Fig. 5]


% cytotoxicity
-/-; med
-/-; IL-2
+/+; med
+/+; IL-2
100/1
50/1
25/1
12.5/1
6.25/1
3.125/1
% cytotoxicity
-/-; IL-18
+/+; IL-18
IFN-γ (ng/ml)
+/+
-/-
N.D.
N.D.
N.D.
med
IL-18

[Fig. 6]


IFN-γ (ng/ml)
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300
200
100
0
+/+
-/-
1st stim.
2nd stim.
(-)
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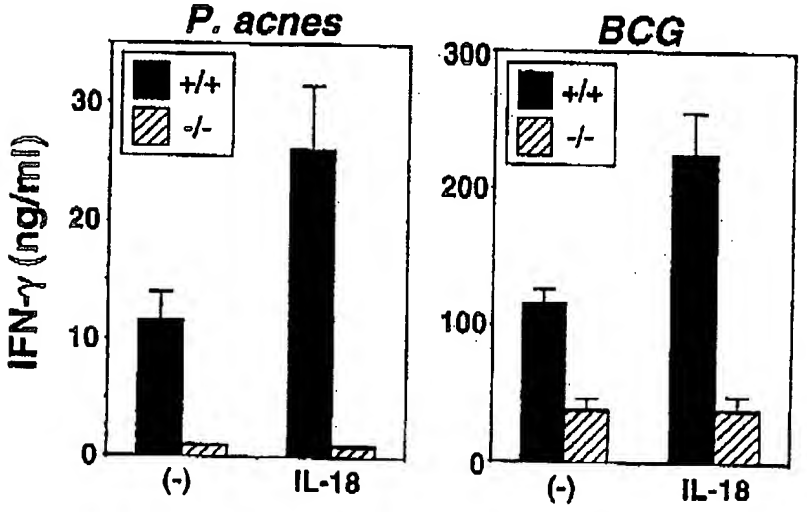
IL-12
IL-12
IL-12
(-)
(-)
IL-18
IL-12
IL-12
+
IL-18


P. acnes
BCG
IFN-γ (ng/ml)
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20
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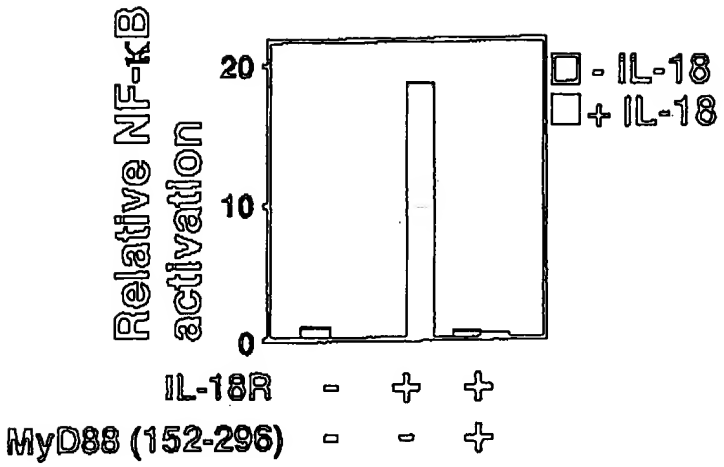
0
300
200
100
0
+/+
-/-
(-)
IL-18
(-)
IL-18

【Fig. 7】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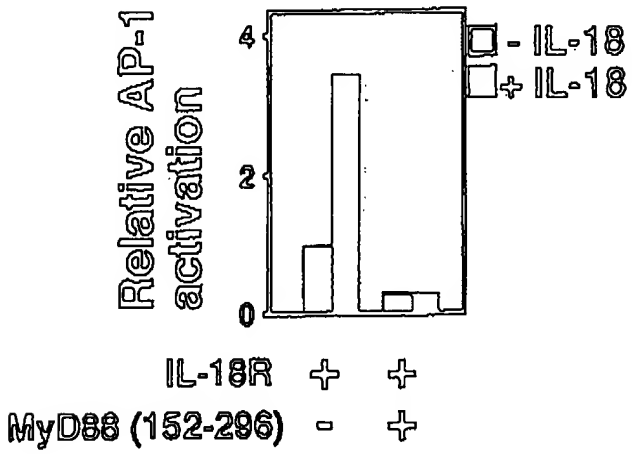
10
0
- IL-18
+ IL-18
IL-18R - + +
MyD88 (152-296) - - +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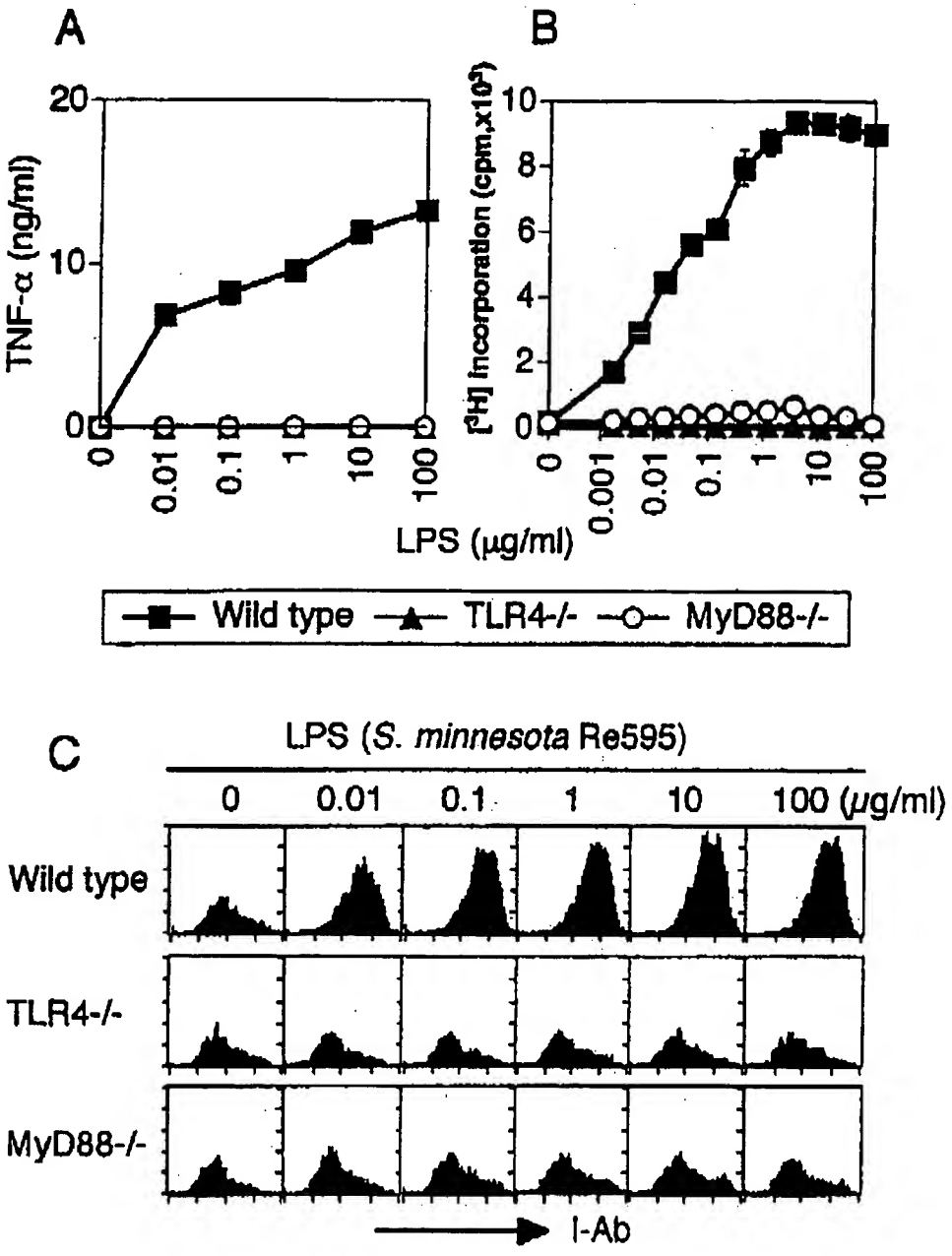
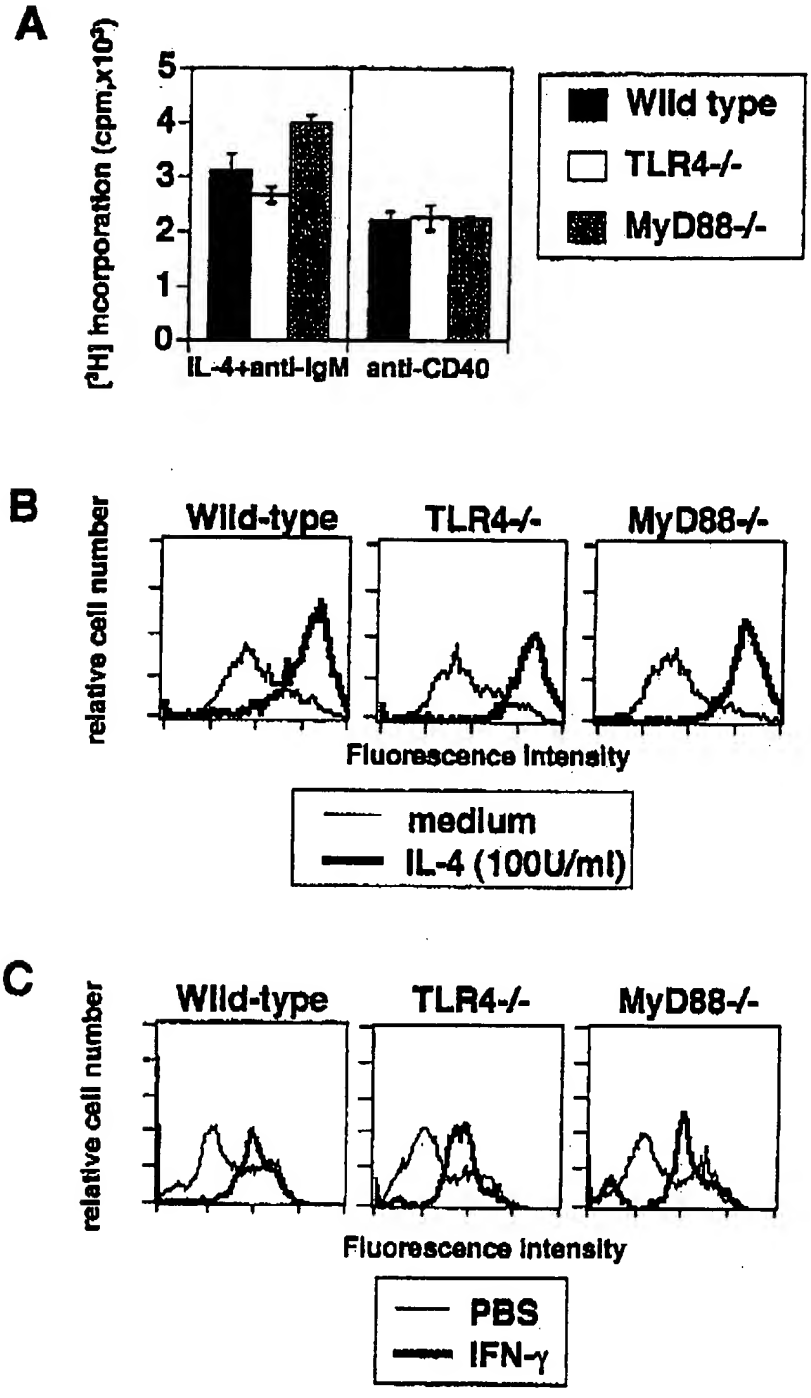
2
0
- IL-18
+ IL-18
IL-18R + +
MyD88 (152-296) - +

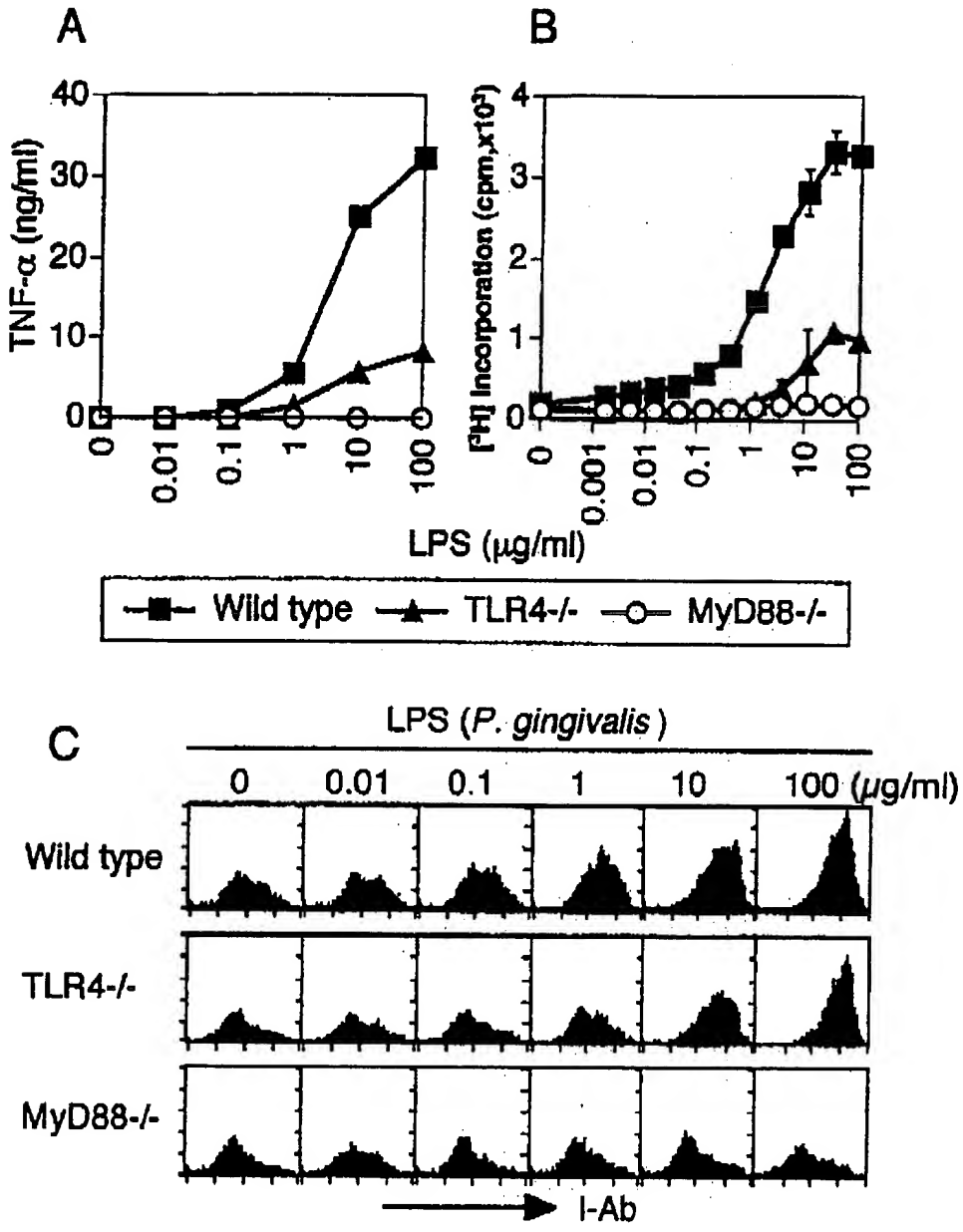
【Fig. 8】


A
B
TNF-α (ng/ml)
[³H] incorporation (cpm,x10³)
LPS (μg/ml)
Wild type
TLR4-/-
MyD88-/-
C
LPS (S. minnesota Re595)
0 0.01 0.1 1 10 100 (μg/ml)
Wild type
TLR4-/-
MyD88-/-
I-Ab

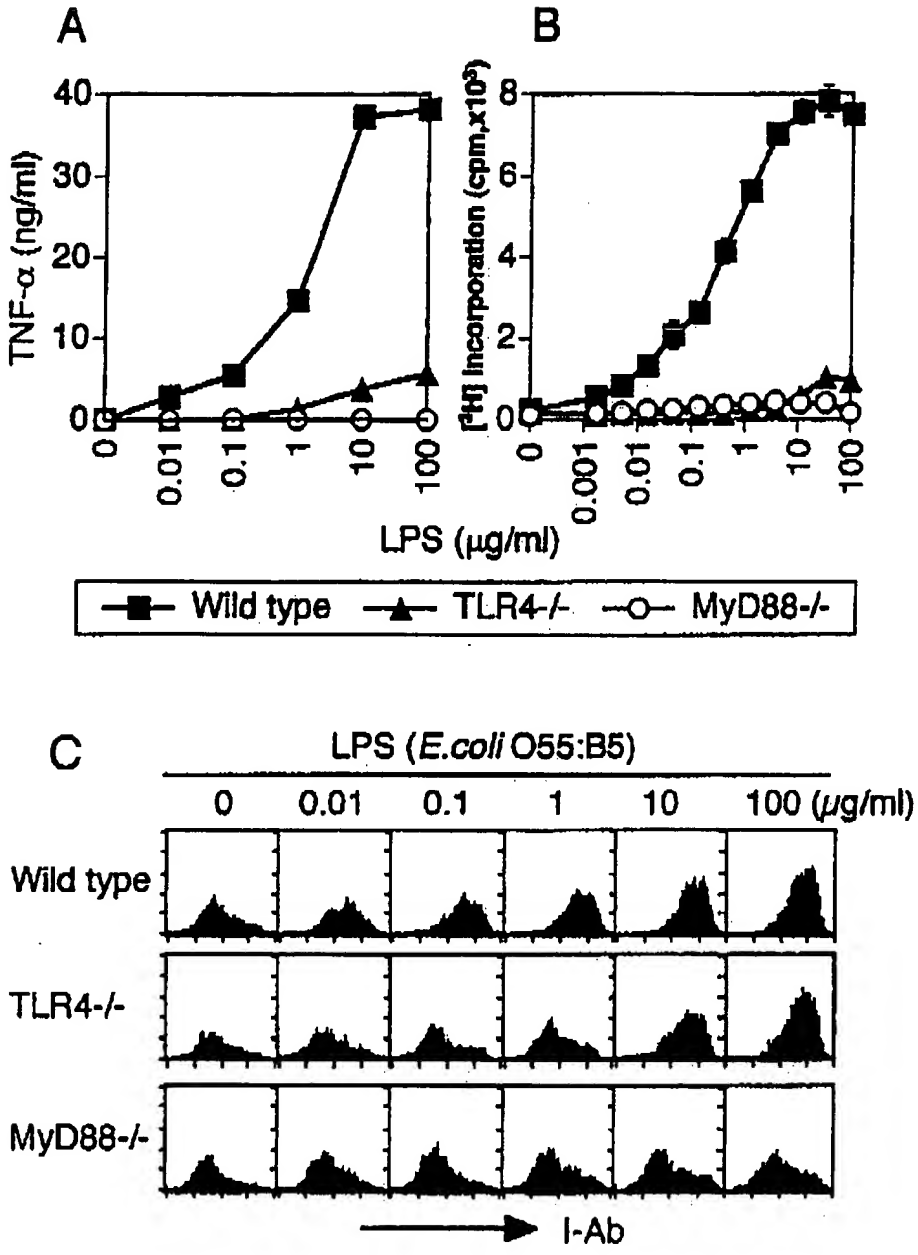
[Fig. 9]


A
[3H] incorporation (cpm,x10³)
5
4
3
2
1
0
IL-4+anti-IgM
anti-CD40
Wild type
TLR4-/-
MyD88-/-
B
relative cell number
Wild-type
TLR4-/-
MyD88-/-
Fluorescence Intensity
medium
IL-4 (100U/ml)
C
relative cell number
Wild-type
TLR4-/-
MyD88-/-
Fluorescence Intensity
PBS
IFN-γ

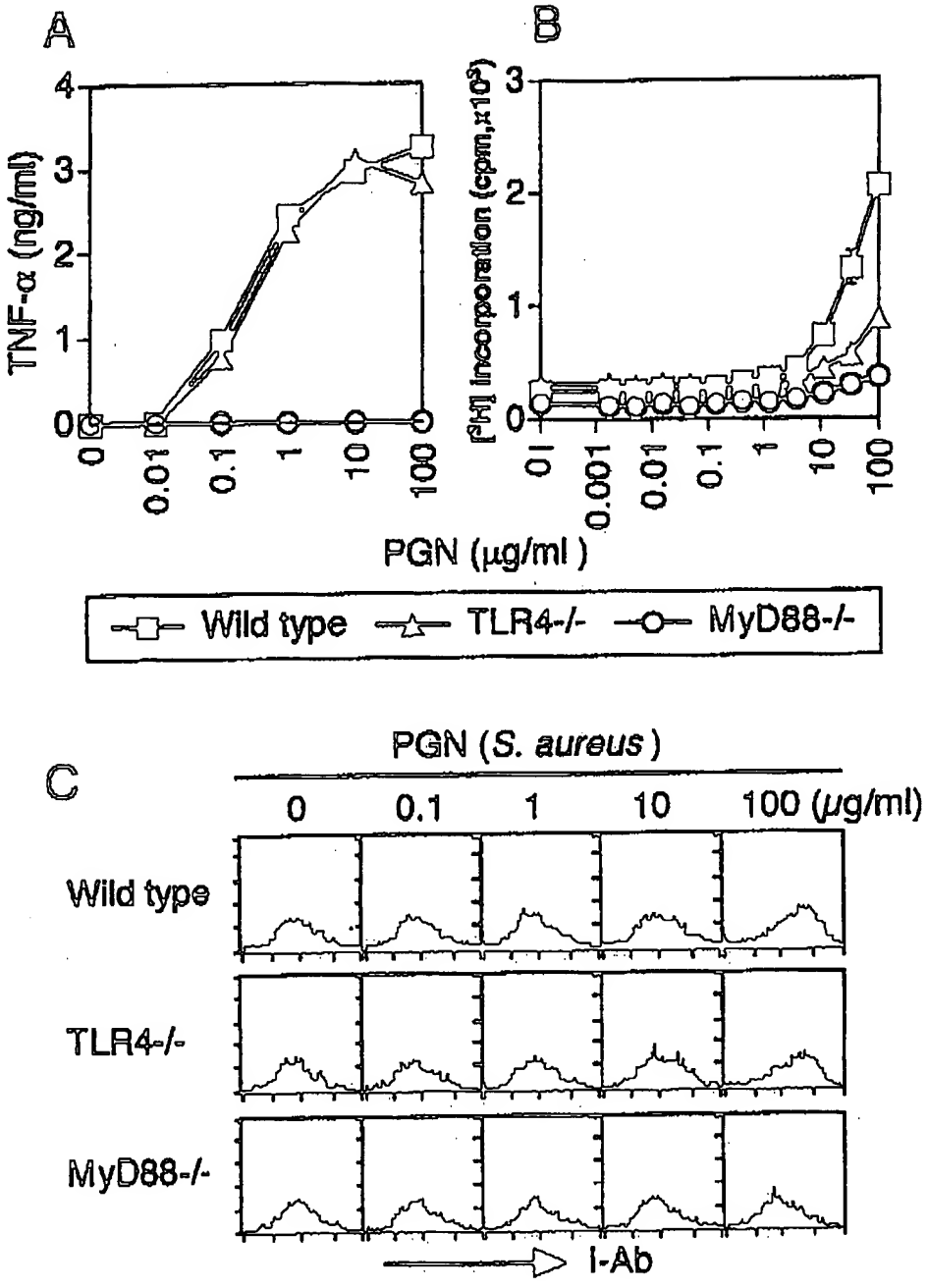
【Fig. 10】


A
B
TNF-α (ng/ml)
[³H] Incorporation (cpm,x10³)
LPS (μg/ml)
Wild type
TLR4-/-
MyD88-/-
C
LPS (P. gingivalis)
0
0.01
0.1
1
10
100 (μg/ml)
Wild type
TLR4-/-
MyD88-/-
I-Ab

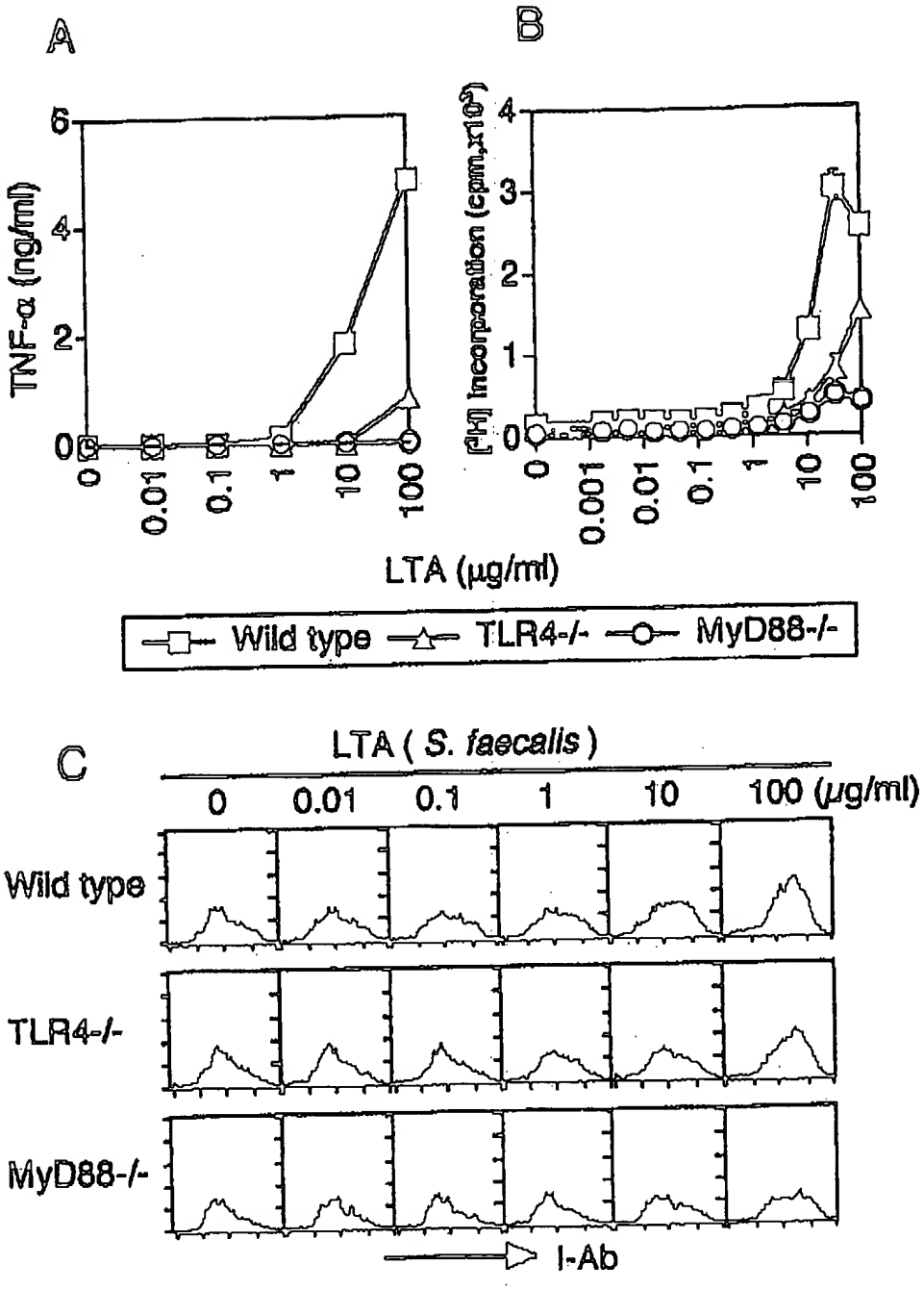
[Fig. 11]


A
B
TNF-α (ng/ml)
[³H] incorporation (cpm,x10³)
LPS (μg/ml)
Wild type
TLR4-/-
MyD88-/-
C
LPS (E.coli O55:B5)
0 0.01 0.1 1 10 100 (μg/ml)
Wild type
TLR4-/-
MyD88-/-
I-Ab

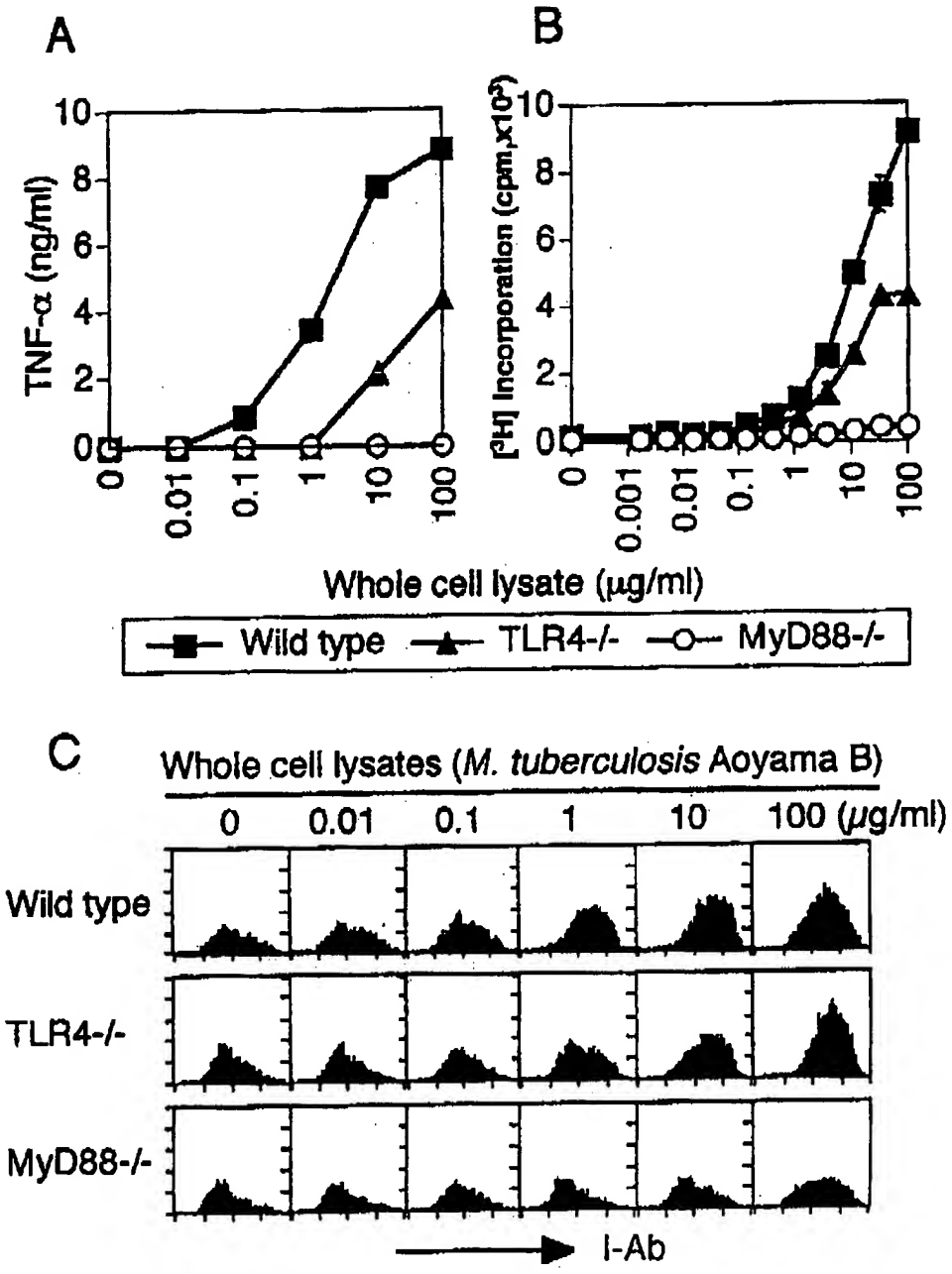
[Fig. 12]


A
B
TNF-α (ng/ml)
[3H] incorporation (cpm, x10^3)
PGN (μg/ml)
Wild type
TLR4-/-
MyD88-/-
C
PGN (S. aureus)
0
0.1
1
10
100 (μg/ml)
Wild type
TLR4-/-
MyD88-/-
I-Ab

[Fig. 13]


A
B
TNF-α (ng/ml)
[3H] incorporation (cpm,x10³)
LTA (μg/ml)
Wild type
TLR4-/-
MyD88-/-
C
LTA (S. faecalis)
0 0.01 0.1 1 10 100 (μg/ml)
Wild type
TLR4-/-
MyD88-/-
I-Ab

【Fig. 14】


A
B
TNF-α (ng/ml)
[³H] Incorporation (cpm,x10³)
0
2
4
6
8
10
0
0.01
0.1
1
10
100
0.001
Whole cell lysate (μg/ml)
Wild type
TLR4-/-
MyD88-/-
C
Whole cell lysates (M. tuberculosis Aoyama B)
0
0.01
0.1
1
10
100 (μg/ml)
Wild type
TLR4-/-
MyD88-/-
I-Ab